SUPPORTING MATERIALS AND METHODS

Colonic Transit Time Assessment

On morning of day 1, patients ingested 24 polyethylene markers (Sitzmark-Konsyl Pharmaceutical, Inc., Edison, NJ) diluted in water. On day 5, abdominal radiographs were obtained with patients in the erect position. The patients were instructed to maintain their regular diet and avoid laxatives and suppositories for the duration of the test.

Anorectal manometry

The manometric study was performed using a stationed pull-through technique with a four-channel water perfused catheter (Mui Scientific, Mississauga, Ontario, Canada) connected to an electronic manometer and to a computer which generated the graphic register (Sandhill Scientific Inc., Littleton, Colorado, USA). Patients were positioned in the left lateral decubitus position. The catheter is placed through the anal canal and advanced into the rectum. A complete manometric evaluation includes determination of the resting pressure, squeeze pressure, the ability of the IAS to relax with straining, the RAIR and the rectal sensation were performed.

Immunohistochemistry and neuronal counts

Three biopsies were placed into a Sylgard-coated Petri dish with ice-cold Hank's balanced salt solution (H4641, Sigma-Aldrich, Italy) and microdissected under a steromicroscope (Leica S6E, Leica Microsystems, Italy) in order to obtain submucosal whole-mounts. These were pinned flat and fixed in 4% paraformaldehyde buffered solution for 3 hours at room temperature. After three washes in phosphate-buffered saline (pH 7.2) solution, submucosal whole mounts were processed for immunohistochemistry.

Each submucosal specimen was incubated for 3h at room temperature in blocking solution containing 2% Triton X-100 and 20% goat serum (Colorado Serum Co., Denver, CO, USA) in

PBS. Tissues were incubated overnight at +4°C in primary antibodies diluted in blocking solution (listed in Supplementary Table 2). Whole mount tissues were then washed in PBS (3 × 10 min) and were then incubated for 2 h at room temperature with the appropriate secondary antibodies (Supplementary Table 2). Finally, after three more washes in PBS, preparations were mounted with buffered glycerol (pH 8.6) and examined on a Nikon Eclipse Ni microscope equipped with the appropriate filter cubes to distinguish the fluorophores employed. The images were recorded with a Nikon DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands). Contrast and brightness were adjusted using Corel Photo Paint, whereas the figure panels were prepared using Corel Draw (Corel Photo Paint and Corel Draw, Ottawa, Canada).

Antibody specificity was assessed by omitting the primary antibody and pre-incubating each primary antibody with an excess of the homologous molecule. Specificity for pChAT and VIP was demonstrated by the lack of immunoreaction in sections in which the primary antiserum was omitted or in sections incubated with primary antibodies pre-absorbed with the corresponding molecule.

RT-qPCR

For each patient one biopsy (10 mg of tissue) was thawed, mechanically disrupted with sterile forceps and homogenized using QIAshredder (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Total RNA Extraction was performed using RNeasy mini kit as indicated and eluted in a final volume of 30 µl. Extracted RNA was purified from genomic DNA via 30 min at 37°C incubation in a thermocycler (Termal Cycler 2027, Applied Biosystem, USA) with a mixture containing the specific *DNasi* enzyme (Thermo Scientific, Milan, Italy) according to manifacturer’s instructions. The product was quantified using a NanoDrop 2000

spectrophotometer (Thermo Scientific, Milan, Italy) and the quality was checked by electrophoresis in 1% agarose.

Each sample (200 ng of total DNA-free RNA in a 20 µl total reaction volume) was reverse transcribed to obtained cDNA using *TaqMan® Reverse Transcription Reagents* (Life Technologies, Thermo Scientific, Milan, Italy). RT cycles were: 15 min 45°C, 3 min 95°C and 5 min 4°C. Resulted cDNA was stored at -20°C.

Relative gene expression analysis was performed on an Applied Biosystem 7500 Fast real time PCR system (Life Technologies, Thermo Scientific, Milan, Italy) by Duplex TaqMan® Gene Expression Assays (Life Technologies, Thermo Scientific, Milan, Italy). Amplification was performed in a 20 µl final volume including 2 µl of cDNA as template and TaqMan® Fast Advanced master mix (Life Technologies, Thermo Scientific, Milan, Italy). Each sample was tested in duplicate and each assay was performed in triplicate. Amplification conditions were: 2 min at 50°C, 20 sec 95°C followed by 45 cycles (30 sec at 95°C, 30 sec at 60°C).

Primer probes were from Applied Biosystem: VIC labelled for *18S* (Hs99999901_S1), FAM labelled for *VIP* (Hs00175021_m1), *VIP receptor 1 – VIPR1*- (Hs00910453_m1), *VIP receptor 2 – VIPR2*- (Hs00173643_m1).

Data are calculated with $\Delta\Delta C_T$ method using *18S* as a reference gene, since the mRNA transcript for this gene was the most reliable in colonic human tissues. The mean value of CTRL group for each gene was the calibrator at unit value.

SUPPORTING RESULTS

Clinical-pathological correlations

We tested whether age, gender, and clinical parameters correlated with changes in TT, AM and VIP and VIPRs expression. In particular, we showed that age and gender did not affect the number of submucosal neurons, TT, AM and VIP or VIPRs gene expression (data not shown). However, in PD/CC, age resulted positively correlated with the neurological and motor scale score UPDRSIII (Spearman's coefficient =.428; *P*= .023; Supplementary Fig. S1 A). The number of HuC/D/VIP-IR neurons resulted negatively correlated with UPDRSIII (Spearman's coefficient =-.694; *P*= .018), i.e. the lower the number of VIP containing neurons, the worst the PD score was (Supplementary Fig. S1 B). The severity of CC in PD (Rome III criteria) was significantly correlated with UPDRSIII (Spearman's coefficient = .624; *P*= .023) (Supplementary Fig. S1 C). Finally TT, i.e. the number of intracolonic residual pellets, was positively correlated with the duration of PD (Spearman's coefficient =.491; *P*= .028) (Supplementary Fig. S1 D).

Supplementary Table 1. Demographic and neurological features of PD/CC patients.

ID	Age (years)	Gender	PDD (years)	UPDRS	UPDRS Part III	HY (1-5)	MMSE	LED (mg)
P1	78	F	2	10	6	1.5	24.7	0
P2	70	M	1	14	7	2	24.9	200
P3	66	M	6	16	10	2	30	200
P4	82	F	3	29	18	3	20	200
P5	68	M	8	18	14	2	26.2	0
P6	82	M	4	19	12	2.5	27	400
P7	83	M	1	39	24	3	23	300
P8	75	M	5	17	10	1.5	27.4	300
P9	72	F	10	10	8	2.5	-	300
P10	64	M	5	15	12	2.5	30	0
P11	85	M	3	19	15	2	16.4	300
P12	78	M	6	13	13	2	24.3	400
P13	65	F	3	4	2	1	30	200
P14	75	F	7	12	12	2	25	400
P15	78	F	-	39	27	2.5	-	-
P16	69	F	6	11	7	2	26.2	200
P17	72	M	7	20	13	2	22.7	375
P18	76	M	2	38	25	3	-	400
P19	82	M	4	14	9	2	23	400
P20	49	M	4	20	24	3	22	400
P21	74	M	-	-	-	-	-	-
P22	61	F	10	17	10	2	-	300
P23	55	M	5	33	25	3	-	550
P24	83	F	6	31	16	2.5	21.5	400
P25	71	M	3	19	13	2	26.7	300
P26	59	M	3	12	9	2	30	400
P27	67	M	2	17	8	2	25.9	200
P28	74	M	4	7	7	2	27.4	700
P29	48	M	5	15	9	2	27	0

Notes: HY, Hoehn & Yahr stage; LED, L-Dopa equivalent daily dose; MMSE, Mini Mental State Examination score; PDD, Parkinson's Disease duration; UPDRS, Unified Parkinson's disease rating scale.

Supplementary Table 2. Details of the primary and secondary antibodies used in the present study.

Primary antibody	**Host**	**Code**	**Dilution**	**Source**
PGP9.5	Rabbit	AB1761	1:500	Merck Millipore
HuC/D	Mouse	A21271	1:50	Life Technologies
VIP	Rabbit	#7913	1:2500	CURE/DDRC[a]
pChAT	Rabbit		1:150	Generous gift of Dr. K. Lips[b]
Secondary antibody	**Host**	**Code**	**Dilution**	**Source**
Anti-mouse IgG Alexa 594	Goat	A11005	1:200	Life Technologies
Anti-rabbit IgG FITC	Goat	401314	1:200	Merck Millipore

Notes: HuC/D, Human neuronal protein; PGP9.5, Protein Gene Product 9.5; VIP, Vasoactive Intestinal Polypeptide; pChAT, peripheral Choline Acetyl-Transferase.

Suppliers: Life Technologies, Termo Fisher, Milan, Italy; Merck Millipore, Merck KGaA, Darmstadt, Germany; [a] CURE/DDRC, DDD, University of California Los Angeles, Los Angeles, California, USA; [b] Justus-Liebig-University, Giessen, Germany.

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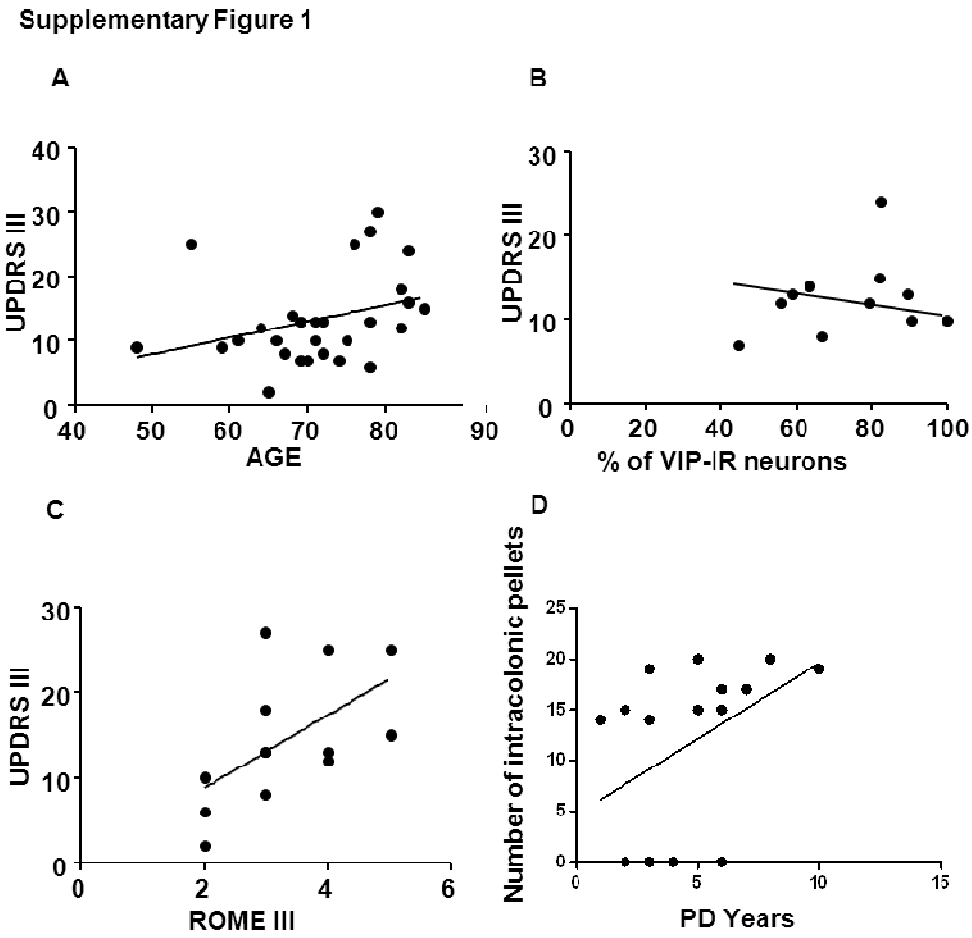

102 **Supplementary Figure S1. Clinical correlations in PD/CC group. (A)** Age resulted positively
103 correlated with the severity of motor impairment (UPDRSIII scale) (Spearman's coefficient
104 =.428; *P*= .023). **(B)** Percentages of VIP containing neurons were negatively correlated with the
105 UPDRSIII scale (Spearman's coefficient =-.694; *P*= .018). **(C)** Rome III severity scale was
106 positively correlated with the UPDRSIII scale (Spearman's coefficient = .624; *P*= .023). **(D)** The
107 number of intracolonic residual pellets (TT) was positively with the duration of PD (years)
108 (Spearman's coefficient =.491; *P*= .028).